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WO 03/037925 A2

(54) Title: PRECURSOR FEEDSTOCK FOR FORMING FILAMENTS

(57) Abstract: The application relates to a method of obtaining silk feedstock solution from a gland of a silkworm or another silk-producing arthropod in which the body of the silkworm or the other silk-producing arthropod is cut open and the gland removed. The silk feedstock solution is subsequently extracted from the gland and used for extrusion, spinning or moulding of objects.

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Title

Precursor Feedstock for Forming Filaments

5 Field of the Invention

This invention relates to feedstock material for spinning of proteinaceous fibres.

Prior Art

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There is currently much interest in apparatus and processes that enable the manufacture of polymer filaments. Strong, tough filaments are useful for the manufacture, by way of example only, of threads, cords, ropes, ribbons and wound, woven or fleece materials. Filaments can also be incorporated into a matrix with or without other filler particles to produce tough and resilient composite materials. Natural silks are fine, lustrous threads produced by the silkworm, *Bombyx mori*, and other insect or arachnid species. Such silks offer distinct advantages when compared with many synthetic polymers currently used for the manufacture of filaments. The tensile strength and toughness of some silks can equal or even exceed that of Kevlar™, one of the strongest of man-made fibres. Silks such as spider dragline also show exceptional thermal stability. Many silks are also biodegradable and do not persist in the environment. They are recyclable and are produced by a highly efficient low pressure and low temperature process using only water as a solvent. The natural spinning process is remarkable in that an aqueous solution of protein is converted into a tough and highly insoluble filament. There are a number of methods both conventional or biomimetic to spin fibres from protein feedstock in order to make fibres. All of these methods rely on synthesized or genetically modified precursor peptide, protein or protein/synthetic feedstock.

20 Natural silk is derived commercially from the silkworm which spins a cocoon that is

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then heated and chemically treated to allow its filaments to soften and unglue making it possible to unravel the cocoon and wind the resulting filament onto a bobbin from which they can then be twisted into threads. The commercial silk industry has a history of many thousand years during which silk worms (*Bombyx mori*) and other

5 lepidopteran species have been bred to produce many races with silks of different qualities. Recently *Bombyx* silk worms have also been genetically modified to give an even wider range of silks. In all cases, however, the spinning of the feedstock is left to the insect itself and thus spinning occurs 'in vivo'.

10 There are many published and known processes and methods to produce silk-like materials or materials derived from silk using not only synthetic feedstock but also recombinant feedstock made from silk sequences expressed for example in bacteria, yeast, vascular plants or even goat milk. It has been suggested that such materials are likely to have highly useful properties. It has also been suggested that the performance

15 of such materials is likely to be improved by matching the extrusion or moulding technology with the properties of the feedstock or vice versa. It will be appreciated that the feedstock solutions required for the manufacture of fibres must have specific properties. There are many patents relating to such feedstock, such as US-A-5,728,810, US-A-5,756,677, US-A-6,268,169 and US-A-6,184,348, which discuss the preparation 20 of synthetic silk protein polymers either by genetic (recombinant) or chemical modification.

There are other inventions discussing the extraction of particular ingredients such as sericin from silk and silk glands for use for example in healthcare and beauty creams.

25 An example of such an invention is disclosed in Chinese patent application Nr. CN-A-1092284 in which a silkworm is cut into fine pieces prior to being soaked in water. According to this patent application, multiple kinds of amino acids are extracted from the silkworm which can be used to make a skin protection product.

30 Prior art disclosing methods of extracting feedstock from silkworms include Japanese Patent Application No. JP-A-6-184809 (Saito) in which the larvae in the final instar are

pressed to death in order to extract the feedstock from their silk glands. Using this method, however, the feedstock is contaminated with body fluids of the silk worm. The removal of these body fluids requires an additional purification step to produce feedstock for spinning or other extrusion methods. Even after purification, some 5 impurities from the body fluids may remain in the feedstock to cause contamination that affects the properties of the final spun material. Moreover, such purifications require addition chemical treatments that in all likelihood seriously degrade the original feedstock.

10 Japanese patent application No. JP-A-2-686693 (Asahi Chemical) describes a method by which silk fibroin is apparently cultivated using rear silk glands in a culture medium.

15 Similarly, Japanese patent application Nr. JP-A-3-209399 (Terumo Corp) teaches a method in which the heads are cut off the silkworms and their abdomina squeezed in order to obtain the silk protein. Again, this method suffers from the disadvantage that not only are silk proteins obtained, but also impurities from the body fluids which requires potentially detrimental chemical removal of the fibroins.

20 Methods for cutting open the silkworm to extract useful materials from the silk worm are described, for example, in Japanese Patent Application Nr. 9-019238 (Toray Industries). This publication discloses an ultrasonic cutter which cuts open the silk worm in order to extract the body fluids, such as interferon. A further method is disclosed in Japanese patent application JP-A-9-070241 (Toray) in which a rotating 25 blade is used to cut open the silkworm.

Summary of the Invention

An object of the present invention is to provide raw silk feedstock material for use in 30 subsequent forming operations by extracting the feedstock material directly from silk glands of silkworms or other silk-producing arthropods. By cutting open the body of the silkworm and removing the gland, followed by extracting the silk feedstock solution,

contamination of the silk feedstock solution by other body fluids is substantially reduced or prevented totally. This means that the silk feedstock solution does not need to be purified prior to use in, for example, spinning or extrusion. It is believed that the best raw silk feedstock material is one that is substantially free from impurities and 5 moreover is little chemically modified from the original feedstock material directly extracted from the glands of the silkworms or other silk-producing arthropods.

The silk feedstock solution can further be advantageously placed in dissolved water for a period of time in order to remove sericin protein in the silk feedstock solution. This 10 leaves a silk feedstock solution that comprises substantially fibroin protein.

In a further embodiment of the invention, the silk feedstock solution is treated with a cross-linking agent, such as an aldehyde. The cross-linking agent introduces covalent bonds between and probably within the protein chains to enhance the mechanical 15 properties of spun or extruded material as well as material formed or poured into shapes as required. One example of this use is in the preparation of a filler, such as for insertion in a bone cavity prepared for an implant such as an artificial hip. After insertion of the peg of the hip, the filler is crosslinked form a firm and perfectly shaped grip linking bone and the implant.

Detailed Description of the Invention

The present invention refers to the direct removal of silk precursor solution directly from the glands of silkworms or other silk producing arthropods. The glands of the silkworm comprise an epithelial layer of tissue consisting largely of a layer of columnar cells surrounded by a basement membrane of structural proteins. Inside the epithelium is a coating of sericin made up of five or so different sericin layers. The coating of sericin surrounds a thick core of fibroin.

5 The precursor solution may be collected and stored and be subjected, if desired, to chemical or physical modification for use as a feedstock for extrusion into filaments or for moulding the material while it sets to produce objects with desired shape. This has the advantage of reducing the labour cost involved in unwinding of cocoons and is likely to produce materials with superior properties. An additional advantage is that,

10 15 unlike the natural spinning process that is extremely difficult to control, the present approach enables rigorous control of the extrusion or moulding process so that the properties of the resulting materials can, in theory, be tuned to the desired end use. By way of example only, the feedstock material of the present invention can be used in methods of forming materials as disclosed in WO 01/38614, the disclosure of which is incorporated in this application. It appears important that for best effect the spinning solution taken from a silkworm is as free from impurities as possible, and also as little chemically modified from the original raw silk solution as possible.

20

25 The invention thus relates to the preparation of silk feedstock solutions by removing aqueous solutions of silk protein directly from the silk glands of silkworms as a precursor from which to extrude or mould materials. It will be appreciated that such raw silk feedstock material can be obtained directly from the silk glands either by dissection or by extraction with a suitable solvent or solvent system as described in more detail hereinafter. The feedstock may be extracted towards the end the final larval instar in order to get a maximum amount the fibroin protein and a minimum amount of the sericin protein. However, the feedstock might be extracted at other larval instarts or

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during other time periods during the final larval instar. The extracted silk protein solutions can be diluted, concentrated, chemically or physically modified, handled and stored in readiness for extrusion or moulding.

5 The extracted silk protein solutions comprise both sericin and fibroin proteins. The Bombyx more fibroin dope can be regarded as a mixture of three proteins the ratio of five molecules of fibroin heavy chain, 5 molecules of fibroin light chain and 1 molecule hexamerin (=P25). It is believed that the hexamerin holds the hexamers formed from the other two units together, hence the 5.5.1 ratio. It is very likely that
10 these have to be assembled into 5.5.1 hexameric units for optimum results.

The fibroin heavy chain protein has been sequenced and deposited under the accession number FBOH_BOMMO (P05790) Fibroin heavy chain precursor (Fib-H) (H-fibroin). It is encoded by the gene FIBH - Bombyx mori (Silk moth). Sequencing is
15 described in the article by Zhou C.-Z., Confalonieri F., Medina N., Zivanovic Y., Esnault C., Yang T., Jacquet M., Janin J., Duguet M., Perasso R., Li Z.-G. "Fine organization of Bombyx mori fibroin heavy chain gene.", published in Nucleic Acids Res. 28:2413-2419(2000).

20 The fibroin light chain protein has been sequenced and deposited under the accession number FBOL_BOMMO (P21828) Fibroin light chain precursor (Fib-L) (L-fibroin). It is encoded by the gene FIBL -Bombyx mori (Silk moth). Sequencing is described in the article by Yamaguchi K., Kikuchi Y., Takagi T., Kikuchi A., Oyama F., Shimura K., Mizuno S. "Primary structure of the silk fibroin light chain determined by cDNA
25 Sequencing and peptide analysis." Published in J. Mol. Biol. 210:127-139(1989). The sequencing is additionally described in Kikuchi Y., Mori K., Suzuki S., Yamaguchi K., Mizuno S., "Structure of the Bombyx mori fibroin light-chain-encoding gene: upstream sequence elements common to the light and heavy chain", published in Gene 110:151-158(1992).

30

A variety of extrusion and moulding techniques can be used to form the final materials

as is commonly known to a craftsman skilled in the art. Specific examples of further treating steps are given in more detail hereinafter.

According to another aspect of the present invention there is provided the use of silk
5 precursor solutions taken from the glands of silkworms or other silk producing
arthropods as feedstock from which to extrude or mould filaments or other structures.
In particular the glands may be taken from silkworm larvae and other lepidoptera or
arachnida. The extracted material can be extruded into high quality fibres using
existing spinning or moulding technologies.

10

By way of example only there follows a method according to which the protein
solution can be extracted. In this method the glands are removed by dissection and the
concentrated spinning solution in them is retained with or without removal of the
epithelial layer by dissection. The glands are removed by slicing open the body of the
15 silkworm or other silk-producing arthropods. The preferred method uses removal of the
epithelial layer after a brief treatment in distilled water. Alternatively, other solvents
could be used. The resulting concentrated protein can be pooled with other protein
extracted in this way and stored, preferably at 4°C, before use. Protease inhibitors may
be added to prevent degradation of the protein during storage as known by persons
20 skilled in the art. A treatment with water or aqueous solution to remove sericin or
other easily soluble compounds may be instituted before or after pooling the glands.
The concentrated protein solution is preferably stored in a siliconized container to
prevent degradation during storage and preferably under silicone oil. The concentrated
protein solution can be used directly or can be diluted with water or other solutions or
25 solvents before use. It can be further treated or used directly. Prolonged storage is
possible after freeze drying the spinning solution before or after dilution.

30 Examples of further treatment steps after extraction of the protein solution involve
treatment with aqueous or non-aqueous solutions or buffers or treatment with vapour
from volatile acids or bases or volatile buffers. A further example is the addition of
mono or divalent ions to condition the spinning solution. These conditioning solutions

can be added to the spinning solution through a semipermeable or porous membrane as described, for example, in WO 01/38614.

5 The solution may be treated by the addition of an aldehyde solution such as glutaraldehyde to cross-link the constituents of the spinning solution. Other aldehydes or cross-linking agents could be used as will be understood by persons skilled in the art. Other conditioning agents can be added to the spinning solution.

10 The final processing of the spinning solution can be achieved in a variety of ways. These include conventional solution or gel spinning techniques; spinning through dies with one or more semipermeable or porous regions; moulding; blow moulding; and blown extrusion. In one embodiment the solution may be allowed to flow into a mould. The solution is then treated with an aldehyde or other cross-linking agent in solution or as vapour applied directly or through a semipermeable membrane forming 15 substantially all or part of the walls of the mould. A similar aldehyde can be applied immediately before the spinning solution enters a blow moulding mould or an annular die used for blown extrusion.

Examples

20 Two illustrative examples of the invention will now be described. These examples are not intended to be limiting of the invention.

Example 1

25 A final instar of the silkworm, *Bombyx mori*, is selected. The instar was approximately 7 cm long and contained a gland having several milligrams of protein mixture. The protein dope contained fibroin and sericin. The gland was removed by dissecting the silkworm. Care was taken to avoid substantial contamination with the silkworm's 30 bodily fluids. The gland was then immersed in distilled water for two minutes.

The epithelium of the gland was removed by knife and the protein dope mixture extracted from the gland. The protein mixture was fixed in a 2% glutaraldehyde solution in a 0.1 M phosphate buffer, having a pH of 7.4 for twelve hours. The resulting cross-linked mixture appeared to have good mechanical properties.

5

Example 2

10 The glands were removed as described above and the epithelium removed. The protein mixture placed into distilled water and agitated for around 30 minutes. The sericin protein dissolved into the distilled water to leave substantially fibroin protein in the protein mixture. The fibroin protein was blotted dry and placed into a mould for storage

JC20 Rec'd PCT/PTO 22 JUN 2005

CLAIMS

1. A method of obtaining silk feedstock solution from a gland of a silkworm or another silk-producing arthropod comprising:
 - a first step of cutting open the body of the silkworm or the other silk-producing arthropod;
 - a second step of removing the gland; and
 - a third step of extracting the silk feedstock solution from the gland.
2. The method according to claim 1, wherein the gland has an epithelial layer which is removed by dissection prior to the step of extracting the silk feedstock solution.
3. The method according to claim 2, wherein the gland is treated with water, preferably distilled water, or another solvent, prior to removal of the epithelial layer.
4. The method according to any one of the preceding claims, in which the silk feedstock solution is treated with a cross-linking agent.
5. The method of claim 4 wherein the cross-linking agent is an aldehyde.
6. Silk feedstock solution obtained from a silkworm or another silk-producing arthropod using the method of one of claims 1 to 5.
7. The silk feedstock solution of claim 6 being substantially made of fibroin protein.
8. The use of the silk feedstock solution of claim 6 or 7 as feedstock from which to extrude or mould objects.
9. Object made from the silk feedstock solution of claim 6 or 7.
10. The object of claim 9 being a filament, ribbon or film.

11. The use of a cross-linking agent to treat a silk precursor solution extracted from the glands of silkworms or other silk-producing arthropods as a feedstock from which to extrude or mould objects or seats for objects.
12. The use of claim 11 wherein the cross-linking agent is an aldehyde.
13. A method of forming spun material by passing a liquid spinning solution through the passage of a die assembly to convert the liquid spinning solution into a solid material, wherein the liquid spinning solution is provided by extracting silk feedstock solution from the glands of silkworms or other silk-producing arthropods according to the method of one of claims 1 to 5.
14. The method of claim 13 further comprising a step of treating the silk feedstock solution prior to passing it through the passage of the die assembly.
15. The method of claim 14 wherein the step of treating the silk feedstock solution comprises the addition of a cross-linking agent.

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European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
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WO 03/037925 A3

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INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	EP 1 241 178 A (NATIONAL INSTITUTE OF AGROBIOLOGICAL SCIENCES) 18 September 2002 (2002-09-18) the whole document	6-8
X	& WO 01 42300 A (NATIONAL INSTITUTE OF AGROBIOLOGICAL SCIENCES) 14 June 2001 (2001-06-14) ---	6-8
X	EP 1 004 699 A (SUMITOMO CORPORATION) 31 May 2000 (2000-05-31) the whole document ---	11,12,15
X	EP 0 352 330 A (TERUMO KABUKISHI KAISHA) 31 January 1990 (1990-01-31) the whole document ---	11,12,15
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the International search

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28/05/2003

Name and mailing address of the ISA

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Masturzo, P

INTERNATIONAL SEARCH REPORT

Int'l Search Application No

PCT/EP 02/12033

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Week 50, 1990 Derwent Publications Ltd., London, GB; AN 1990-372015 XP002238659 "Silk fibroin preparation by cultivation of silk thread gland of silkworm" & JP 02 268693 A (ASAHI CHEMICAL INDUSTRIES), 2 November 1990 (1990-11-02) cited in the application abstract</p> <p>---</p>	1-8
X	<p>DATABASE WPI Week 30, 1991 Derwent Publications Ltd., London, GB; AN 1991-314191 XP002238660 "preparation of fibroin natural protein by excising silk threads from adult silkworms and eliminating sericin" & JP 03 209399 A (TERUMO CORPORATION), 12 September 1991 (1991-09-12) cited in the application abstract</p> <p>---</p>	1-8
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 KNIGHT, D. P. ET AL: "Liquid crystals and flow elongation in a spider's silk production line." retrieved from STN Database accession no. PREV199900203151 XP002238657 & PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON SERIES B, (MARCH 7, 1999) VOL. 266, NO. 1418, PP. 519-523.,</p> <p>abstract</p> <p>---</p>	13,14
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; VOLLRATH, F. (1) ET AL: "Silk production in a spider involves acid bath treatment." retrieved from STN Database accession no. PREV199800319921 XP002238658 & PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON SERIES B BIOLOGICAL SCIENCES, (MAY 7, 1998) VOL. 265, NO. 1398, PP. 817-820.,</p> <p>abstract</p> <p>---</p>	13,14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/12033

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 9-10 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9-10

Present claims 9-10 relate to a product defined by reference to a desirable characteristic or property, namely to be composed by a material obtained by the process described in the previous claims.

The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for none of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for claims 9-10.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report	Publication date		Patent family member(s)	Publication date
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JP 2268693	A 02-11-1990		NONE	
JP 3209399	A 12-09-1991		NONE	

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